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Homeobox genes and patterning of the proximal-distal axis in regenerating limbs

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We have examined expression of HoxA genes, and of Msx-2 in developing and regenerating axolotl limbs, and in the developing lateral line system. We have found that Hox complex genes are regulated differently in limb development and regeneration. In development, Hoxa-9 and Hoxa-13 follow the rules of spatial and temporal colinearity seen in other developing limbs. However, in regeneration expression of both Hox genes occurs simultaneously and in the same physical location. The expression pattern is the same regardless of amputation level. Expression is initiated within a day of amputation, and the genes are expressed by the cells of the mature limb, days before these cells dedifferentiate to form a blastema. Spatially distinct domains of expression, identical to those in developing limbs, emerge during growth of the blastema. Factors from the wound epidermis may control reexpression of HoxA genes, and expression is affected in a position specific manner by retinoic acid. Finally, we have also studied the expression of Msx-2 in regenerating limbs and in the lateral line system. In regenerating limbs, Msx-2 is expressed prior to wound healing. In the lateral line system, Msx-2 is expressed continuously from placodal stages through to the mature neuromast.

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FINAL REPORT

Grant#: N00014-92-J-1967 R&T Code: 3414-218

PRINCIPAL INVESTIGATORS: Dr. S. V. Bryant & Dr. D. M. Gardiner

INSTITUTION: University of California, Irvine

GRANT TITLE: Homeobox genes and patterning of the proximal-distal axis in regenerating limbs

AWARD PERIOD: 1 June, 1992 - 31 May, 1996

OBJECTIVE: To investigate the roles of homeobox genes of the *HoxA* complex and *Msx-2* in patterning of the proximal-distal axis during limb regeneration in axolotls.

APPROACH: Axolotls are grown to about 6 cm, and then their forelimbs are removed to induce regeneration. Axolotls at different stages of development and regeneration are collected and analyzed by Northern blots, *in situ* hybridization, PCR, or immunohistochemistry. Effects of growth factors and retinoic acid (RA) are studied by implanting beads. The roles of the epidermis and nerves in gene expression are tested. Cell lines are established for use in functional analysis.

ACCOMPLISHMENTS: During this grant period we have perfected the technique of whole mount in situ hybridization for developing and regenerating axolotl limbs, and have compared the expression of two members of the *HoxA* complex: *HoxA13* and *HoxA9*. The surprising result of this study is that these genes are regulated differently in development and regeneration. In developing limbs the genes follow the rules of spatial and temporal colinearity previously described for developing mice and chickens. However, in regeneration, spatial and temporal colinearity are abrogated and expression of both *Hox* genes occurs simultaneously and in the same physical location. Further, the expression pattern is the same regardless of the position at which the limb was amputated. Another surprise was that expression is initiated within a day of amputation, and that the genes are expressed by the cells of the mature limb, days before these cells dedifferentiate to form a blastema. Expression of the *HoxA* genes continues to be co-localized through the early stages of blastema formation. Spatially distinct domains of expression emerge during growth of the blastema as *HoxA13* expression becomes confined to a distal subset of cells that also express *HoxA9*. At these later stages of regeneration, the expression patterns resemble those of developing limbs. We have begun to study factors that can alter the expression of these *Hox* genes. When the amputation surface is covered with full thickness skin, expression of both genes is suppressed, suggesting that factors from the wound epidermis may control reexpression of *HoxA* genes. When amputation is made through the base of the digits, expression is absent in all regions where the epidermis remains, lending support to the conclusion that the wound epidermis acts locally to control reexpression. Using a cell line we have established, we have found that more than a dozen *Hox* genes (including *HoxA13* and *A9*)

are expressed *in vitro*, and the expression of many is altered in response to components of the medium. Finally, we have found that axolotl *HoxA* genes show similar responses to RA to those described for teratocarcinoma cells: the most 5' genes (*HoxA13*) are inhibited by RA, whereas more 3' genes (*HoxA9*) are either unaffected or are upregulated. When the most 5' gene of the *HoxA* complex is inhibited, the *Hox* code of the affected cells is shifted to that of a more proximal limb level. This shift in *Hox* code is consistent with the biological effect of RA on regenerating limbs, which is to cause a shift in the positional identity of the affected cells to that of a more proximal level. This result also provides independent confirmation that HoxA genes are involved in specification of identity along the proximal-distal limb axis. Confirmation of this type is essential in this system where direct tests of function have not yet become technically feasible.

During the period of this award we have also studied the expression of *Msx-2* in both regenerating limbs and in the lateral line system. In regeneration, we have found that *Msx-2* is expressed very early, prior to wound healing, and its expression is common to both wound healing and regeneration. In the lateral line system, *Msx-2* is expressed continuously from the first detectable epithelial placode through to the mature neuromast. *Msx-2* expression in the lateral line is unique in being exclusively expressed in the epidermis, and in not being associated with epithelial-mesenchymal interactions. Another unique finding in neuromasts, is that *Msx-2* transcripts are localized in the cytoplasm of the support cells on the side closest to the sensory hair cells. The appearance of whole mount preparations suggests that *Msx-2* expression is most intense in cells that are differentiating into sensory cells, possibly as a result of injury followed by regeneration.

SIGNIFICANCE: Regeneration offers an opportunity to look at gene regulation from a unique perspective, one in which genes are reactivated in the mature animal, rather than as part of a developmental cascade that begins at fertilization. Although we are just at the beginning of a molecular understanding of regeneration, it is already clear that regeneration is similar in broad details to development, and that the same genes are involved in both processes. However, the way in which expression of these genes is controlled is different, at least in the case of the *Hox* complex genes we have examined so far, and in all likelihood for other genes too. Herein may lie the key to making regeneration a therapeutic possibility; we need to understand these alternative gene control strategies in order to be able to initiate regeneration in higher vertebrates including humans. It will also be important to understand the regulation of those genes that are at the head of the regeneration cascade, because it is possible that if these genes could be activated, then regeneration could be induced in humans.

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